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(54) Title: BRIDGING ANTIBODY FUSION CONSTRUCTS

(57) Abstract

Disclosed is a bridging antibody construct including a heavy chain variable region which, when combined with a light chain variable region, binds to a surface antigen on an effector cell; a heavy chain constant region comprising a C_{H3} domain; and a non-immunoglobulin binding agent which binds a surface protein on a target cell. The binding agent is peptide-bonded to the carboxy terminus of said C_{H3} domain. Also disclosed are a nucleic acid sequence encoding the construct, a cell line transfected with that nucleic acid, a method of producing the construct, and methods of selectively killing a target cell using the construct.

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BRIDGING ANTIBODY FUSION CONSTRUCTS

This application is a continuation-in-part of the copending United States patent application Serial No. 07/612,110, filed November 9, 1990.

BACKGROUND OF THE INVENTION

This invention relates to therapies involving selective destruction of cells *in vivo*, and more specifically, to compositions of matter useful in the treatment of various cancers and viral infections. In particular, this application relates to genetically engineered-antibody fusion constructs capable of targeting an infected cell and bringing that cell into contact with an effector cell which can kill or neutralize its detrimental activities.

Hormone receptors have been used as tumor-specific markers for the delivery of cytotoxic agents to tumor cells. For example, *Pseudomonas* exotoxin and *diphtheria* toxin have been coupled to peptide hormones and have been shown to be highly cytotoxic and specific for receptor-bearing cells (Astan et al. (1989) J. Biol. Chem. 264:15157-15160; Bacha et al. (1988) J. Exp. Med. 167:612-622).

Antibodies have been shown to mediate the lysis of tumor cells *in vitro* by bridging the Fc receptor (FcR) on the cytotoxic effector cell and the antigenic site on the target cell (Henkart (1985) Ann. Rev. Immunol. 3:31-58. The binding is mediated by the variable (V) regions of the heavy (H) and light (L) chains of the anti-tumor cell antibody and the FcR binding site on the constant (C) region of the Ig H chain. In an analogous manner, cytotoxic T lymphocytes have been targeted to cells for which they have no natural specificity

through the use of cross-linking agents. These include several hetero-bifunctional reagents that share the same mechanism; they bridge a specific marker on the tumor cell surface to a component of the T cell receptor (TCR) and in this way activate the lytic program of the cytotoxic T lymphocyte (Lui et al. (1985) Proc. Natl. Acad. Sci. USA 82:8648-8652; Perez et al. (1986) J. Expt. Med. 163:166-178; Jung et al. (1986) Proc. Natl. Acad. Sci. USA 83:4479-4483).

However, the use of heterobifunctional antibodies and chemical cross-linking reagents may not be efficient. Because of the random association of multiple H and L chains, only a fraction of the resulting antibodies usually are active. Similarly, the binding of a chemical cross-linking reagent may disrupt or inactivate the site or protein at which the reagent binds and hence may not enable the triggering of the effector cells' killing or neutralizing activities.

Among the targeting approaches used to combine anti-T cell and anti-tumor cell specificities is the biochemical conjugation of a peptide hormone to an antibody which recognizes a surface antigen on a receptor-bearing cell (see, e.g., Lui et al. (1988) Science 239:395-398). This approach has some advantages over hetero-bifunctional antibodies. First, the random association of the multiple H and L chains is avoided, resulting in a more homogeneous preparation. Second, the targeting of hormone receptors, relative to other tumor-associated antigens, may lead to the preferential killing of those cells that overexpress the hormone receptor (i.e. the most rapidly growing cells) and thus, are the most malignant.

Therefore, what is needed is an alternative targeting approach involving the use of a heterobifunctional antibody/ligand conjugate or construct that physically bridges a receptor-bearing tumor target cell and an effector cell, and

that activates the killing mechanism. Using this approach it should be possible to confer upon a population of effector cells an anti-tumor specificity that it does not normally have and would lose as soon as the construct is withdrawn or metabolized in vivo. Thus, such a construct would be useful in an adoptive immunotherapeutic approach either alone or in conjunction with the administration of a patient's activated effector cells.

Accordingly, an object of the invention is to provide a construct that bridges an effector cell and a target cell, thereby enabling the killing or the neutralization of that target cell. Another object is to produce a bridging construct that will not inactivate the killing or neutralizing activities of the effector cell when it is bound thereto. Yet another object is to provide an efficient and effective method of targeting effector cells to malignant or virus-infected cells. Still another object is to provide a method of producing these bridging constructs.

SUMMARY OF THE INVENTION

Using the genetic approach, antibody fusions constructs have been produced which effectively bridge a target cell, such as a malignant or virus-infected cell, and an effector cell. Such constructs enable treatment of malignancies and virus infections with accuracy and efficiency.

A representative antibody fusion construct includes a heavy chain variable region, a heavy chain constant region having a C_{H3} domain, and a non-immunoglobulin binding agent which binds a surface antigen or receptor on a target cell. The heavy chain constant region may also include other domains such as a C_{H1} domain and/or C_{H2} domain. The heavy chain variable region, when combined with a light chain variable region, binds to a surface antigen on an effector cell. The binding

agent can be a ligand or a receptor.

The term "nonimmunoglobulin binding agent" as used herein refers to a protein or polypeptide including ligands, receptors, or single chain binding sites that mimic antibody binding sites with predetermined specificity for a surface antigen on a target cell.

The term "effector cell" as used herein refers to any cell which can neutralize or destroy the target cell with which it has been placed in contact. The invention takes advantage of the existence of particular surface proteins or antigens which are specific for a particular class of effector cells.

One preferred construct includes a heavy chain variable region having specificity for the CD3 antigen found on the surface of cytotoxic T lymphocytes. Other constructs embraced by the invention have heavy chain variable regions with specificities for a particular surface antigen on other effector cells such as macrophages, monocutes, natural killer cells, eosinophils, and large granular lymphocytes.

In one aspect of the invention, the non-immunoglobulin binding agent includes a hormone or a growth factor which binds a receptor specific for that ligand. One preferred growth factor is an epidermal growth factor (EGF), or an analog or fragment thereof, capable of binding the EGF receptor found on a target cell.

In another aspect, the non-immunoglobulin binding agent is a receptor which recognizes and binds a surface protein on a virus-infected cell such as an HIV-infected cell. For example, one construct includes a CD4, or an analog or fragment thereof, which is capable of binding the gp120 envelope protein.

In yet another aspect, the non-immunoglobulin binding agent is a single chain binding site, as for example a peptide sequence derived from a mammalian antibody specific for an antigen which is characteristic of a particular target cell.

This invention also embodies nucleic acid sequences such as DNA or RNA encoding the amino acid sequence of a bridging antibody construct, as well as cell lines transfected with such nucleic acid sequences which produce the aforementioned construct. Preferred cell lines to be transfected are myeloma and hybridoma cell lines.

In addition this invention encompasses methods of producing the bridging antibody constructs as well as methods of selectively killing a target cell in vivo with the use of these constructs

The bridging antibody constructs may be prepared as follows. Nucleic acid sequences encoding amino acid sequences of a heavy chain variable region, a heavy chain constant region, and a non-immunoglobulin binding agent, are linked. A host cell is transfected with this nucleic acid and cultured such that it expresses the construct. The host cell may be transfected concurrently with a nucleic acid sequence encoding a light chain variable region. The expressed heavy chain variable region/ligand construct and the expressed light chain variable region may then be combined to form a two or four chain construct.

Moreover, a target cell may be selectively killed in vivo by preparing a bridging antibody construct specific for that target cell and for an effector cell capable of killing or neutralizing that target cell, and then administering the construct to the circulation of a subject harboring the target cell.

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a schematic representation of one embodiment of the bridging antibody construct of the present invention;

FIG. 2 is a diagrammatic representation of the construction of an antibody fusion construct including the human Cγ1 Ig heavy chain and EGF. FIG. 2A is the restriction map of a Cγ1 gene fragment cloned in plasmid pBR322. FIG. 2B shows the fusion of the Cγ1 gene at the Sma I site to a synthetic EGF-encoding sequence. FIG. 2C shows the sequence at the junction of the Ig C_{H3} domain and the amino terminus of EGF;

FIG. 3 is a graphic representation of EGF receptor binding activity of the anti-CD3/EGF conjugate. The activity is measured by comparing the abilities of the conjugate, cold EGF, and anti-EGF receptor antibody to compete with labelled EGF for EGF receptors on M-24 melanoma cells;

FIG. 4 is a graphic representation of anti-CD3/EGF conjugate-induced killing of tumor cell A431 epidermal carcinoma cells (FIG. 4A), M24 metastatic melanoma cells (FIG. 4B), and IMR-32 neuroblastoma cells (FIG. 4C), by TIL 660 cells;

FIG. 5 is a graphic representation of anti-CD3/EGF

conjugate-induced killing of A431 (FIG. 5A) and M24 (FIG. 5B) cells by peripheral blood-derived cytotoxic T lymphocytes. Killing assays were carried out as in FIG. 4;

FIG. 6 is a diagrammatic representation of the preparation of an antibody fusion construct including the human Cγ4 chain and a single chain binding site, in which FIG. 6A shows details of a V_L-linker-V_H sequence and FIG. 6B illustrates an assembled expression vector pdHL2-αCD3/sca-X; and

FIG. 7 is a graphic representation of anti-CD3/single chain binding site conjugate-induced killing of M21 melanoma cells by TIL 660 effector cells, using the construct described in connection with FIG. 6.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns bridging antibody, constructs useful for homing an effector cell to a malignant or virus-infected target cell. The construct includes a conjugate of an antibody portion having a specificity for a surface antigen on an effector cell, and a non-immunoglobulin binding agent complementary to receptors or ligands found on the target cell.

The immunoglobulin portion includes a heavy chain variable region (V_H) which, when combined with a light chain variable region (V_L), binds to a surface antigen on an effector cell. It also includes at least a heavy chain C_{H3} domain peptide-linked to the carboxy terminus of the V_H domain. C_{H1} and/or C_{H2} domains may also be peptide-linked to the carboxy terminus of the V_H domain and to the amino terminus of the CH_3 domain. Without the C_{H1} and/or the C_{H2} domains, the half-life of the construct decreases in vivo. The immunoglobulin portion of the construct may be chimeric in that the variable region may

come from one species and the constant region from another.

FIG. 1 shows a schematic view of a representative bridging antibody construct 10. In this embodiment, ligand molecules 2 and 4 are peptide bonded to the carboxy termini 6 and 8 of C_{H3} regions 10 and 12 of antibody heavy chains 14 and 16. V_L regions 26 and 28 are shown paired with V_H regions 18 and 20 in a typical IgG configuration, thereby providing two antigen binding sites 30 and 32 at the amino ends of construct 10 and two receptor-binding sites 40 and 42 at the carboxy ends of construct 10. Of course, in their broader aspects, the constructs need not be paired as illustrated.

A particularly useful specificity for the V_H region 26 or 28 is that for CD3, a closely associated component of the T cell receptor found on cytotoxic T lymphocytes (CTLs). CTLs lyse the cells to which they are targeted. The construct can thus induce CTLs to kill tumor cells or virus-infected cells for which they bear no specificity. Specificity for other known surface antigens found exclusively or mostly on other effector cells, such as monocytes, macrophages, natural killer cells, eosinophils, or large granular lymphocytes, also may be useful. Monoclonal antibodies to such cell surface structures are known in the art and can be generated using known techniques.

Binding agents include non-immunoglobulin molecules such as ligands and receptors. Useful ligands include those molecules complementary to receptors or surface proteins on the chosen target cell. Useful ligands include hormones such as melanocyte stimulating hormone (MSH), among many others. Alternatively, the ligand may be a growth factor or other non-immunoglobulin preferably single-chain polypeptide which can bind to a receptor on a target cell.

One particularly useful ligand includes epidermal growth

factor (EGF) because a number of malignant cells are known to overexpress EGF surface receptors. In fact, enhanced EGF receptor expression has been known to lead to increased tumorigenicity. In addition, enhanced EGF receptor expression may also serve to discriminate malignant cells from their normal cell counterparts.

A particularly useful binding agent is a receptor such as a CD4 which binds the gp120 envelope protein or HIV, and also is capable of binding the same protein expressed on the surface of HIV-infected cells.

Other binding agents include single chain binding sites which mimic the antibody binding site including V_H and V_L domains as disclosed in U.S. Patent No. 4,946,778 (Ladner et al.) and International Application No. PCT/US88/01737 (Creative BioMolecules, Inc.), published December 1, 1988.

The binding agents may be whole native or synthetic molecules or fragments which retain the ability to bind their receptor. They may have the same amino acid sequence of the native form of the ligand, or instead may be an analog of the native form of the ligand having an amino acid sequence sufficiently duplicative of the native sequence such that the analog binds the native receptor on the target cell.

These constructs are produced by known recombinant DNA technologies including the preparation of a nucleic acid sequence encoding an amino acid sequence for the antibody/binding agent construct, transfecting a host cell line with that nucleic acid, and then culturing the transfected cell line to produce the construct.

Briefly, a gene encoding the non-immunoglobulin ligand, or fragment or analog thereof, is ligated into a plasmid capable of transfecting a preselected host cell for

expression. This gene fragment may be prepared by any number of known techniques. For example, DNA encoding the ligand may be synthesized from the known amino acid sequence of the ligand, or may be obtained from an established cDNA library.

The nucleic acid sequence of native EGF is known (see, e.g., Gregory et al. (1977) J. Peptide Protein Res. 9:107-118) and shown in SEQ ID NO:1. Alternatively, the sequence of any number of known EGF analogs may be used (see, e.g., GB patent application no. 2210618; and Patent Cooperation Treaty Patent Application No. WO 89/1489A, herein incorporated as reference).

The nucleic acid sequence for CD4 (also known as T4) is known (see, e.g., Maddon et al. (1985) Cell 92:93-104), and shown in SEQ ID NO:2. In addition, the nucleic acid sequence of any number of analogs or fragments of CD4 can be used (see, e.g., Patent Cooperation Treaty Application Nos. WO 90/01870A and WO 90/00566, herein incorporated as reference).

DNA encoding immunoglobulin light or heavy chain variable and constant regions is known and is readily available from cDNA libraries or is synthesized biochemically (see, e.g., Gillies et al. (1989) J. Immunol. Meth. 125:191-202; Morrison et al. (1984) Ann. Rev. Immunol. 2:239-256; Falkner et al., (1982) Nature 298:286-288; and Adams et al. (1980) Biochem. 19:2702-2710).

Host cells are transfected by any number of known transfection techniques such as spheroplast fusion (Gillies et al. (1989) Biotechnol. 7:799-804), and then cultured to express the foreign DNA. The host cells transfected may be prokaryotic or eucaryotic. However, if prokaryotic host cells are used, the construct produced must be processed or folded after purification from the cells. Eucaryotic host cells are preferred, as the protein produced therein may be processed by

the cell once it is translated. Particularly useful eucaryotic host cells include myelomas and hybridomas such as non-producing hybridomas (e.g., Sp2/0) and non-producing myelomas (e.g., X63Ag8.653). These host cells may be transfected with more than one nucleic acid sequence such as a nucleic acid encoding the light chain variable region in addition to one encoding the construct. Constructs synthesized by a myeloma or hybridoma cell may be paired with a light chain variable region or an entire light chain within the cell.

The construct is then purified from the cytoplasm of the host cells or from the culture media, depending on the nature of the host cells used. Protein purification methods are numerous and include various chromatographic methods.

Other methods of producing the construct are, of course, possible including the preparation of an RNA sequence encoding the construct and its translation in an appropriate in vivo or in vitro system.

These genetically-engineered constructs have many uses. For example, constructs of the invention can be used to kill selectively a target cell in vivo. One prepares a construct with the specificities of choice, and then administers a therapeutically effective amount to the circulatory system of a subject harboring the target cell. The construct may be administered in physiologic saline or any other biologically compatible buffered solution which will not affect the ability of the construct to bind the effector and target cells. This solution may be administered systemically via IV or by intramuscular injection. Alternatively, the construct may be administered by injection directly at the site to be treated. A truncated construct not having a C_{H1} and/or C_{H2} domain may be useful for this purpose as its half-life is limited in vivo.

The construct also may be used to treat cells in vitro

which then may or may not be returned to a subject. For example, effector cells may be removed from a subject, treated by incubation with the construct to bind thereto, and then returned to the subject where the effector cell/construct conjugate is targeted to a target cell for killing or neutralizing.

Constructs comprising anti-T cell antibodies and peptide hormones are useful in testing the feasibility of adoptive immunotherapy whereby a patient's tumor-infiltrating lymphocyte (TIL) cell line or peripheral blood-derived cytotoxic T lymphocyte line is given an additional target specificity. In particular, since many different tumors overexpress the EGF receptor, the use of conjugates containing EGF is particularly useful for many different cancers.

The ability of an EGF-containing construct to bind the EGF receptor was examined in a competitive binding assay. FIG. 3 shows EGF receptor binding activity of a construct including an immunoglobulin moiety with anti-CD3 specificity and EGF as the ligand moiety. The ability of the construct (Δ - Δ) to compete with labeled EGF for its receptor was measure using M24 melanoma cells as target cell, and compared to unlabeled EGF (o - o), unconjugated anti-CD3 antibody (-) and anti-EGF receptor antibody 225 (◇ - ◇). The results are normalized to the molar equivalents of EGF. The anti-CD3 antibody alone showed little or no inhibition activity while the anti-CD3/EGF construct competed well with EGF for its receptor.

A population of TIL cells derived from a patient with a malignant melanoma was used as a source of activated T-cells for testing a genetically engineered anti-T cell/EGF construct. These cells had little or no cytolytic activity against the tumor targets against which they were tested. In the presence of very low concentrations of the conjugate,

cells expressing EGF receptor were killed readily. This activity was seen at concentrations (10^{-12} to 10^{-11}M) that were significantly lower than the K_D for EGF binding to its receptor ($2 \times 10^{10}M^{-1}$).

A second cytotoxic T lymphocyte line, derived from peripheral blood and specific for autologous Epstein Barr Virus (EBV)-transformed cells but having no specificity for tumor cells, also can be induced to kill the tumor cells. These lymphocytes have been maintained in culture for an extended time in the presence of IL-2 and stimulated bimonthly with mitomycin C-treated autologous EBV-transformed B cells. The ability of these cells to kill EGF receptor-bearing tumor cells over an extended period has not diminished, thus making this EBV-specific cytotoxic T lymphocyte system generally useful for testing hormone constructs.

The specificity of a construct of the present invention was examined by testing the activity of the anti-CD3 antibody alone or in combination with unconjugated EGF. The results which follow clearly demonstrate that the two need to be physically linked for activity.

The epidermal carcinoma cell line, A431, expresses a very high number (2×10^6/cell) of EGF receptor on its cell surface, and this overexpression has been correlated with its ability to form tumors in nude mice (Santon et al. (1986) Cancer Res.46: 4701- 4705). The ability of the anti-CD3/EGF construct to mediate the killing of labeled A431 cells by a human TIL cell line (TIL 660) in a 4 hour chromium release assay was tested, and the results are shown in FIG. 4A. ^{51}Cr-labeled targets were incubated for four hours with the indicated amount of construct and varying ratios of effector cells. The amount of released radioactivity was used to calculate the percent of target cell lysis.

The parameters that were varied in the first studies were the effector cell-to-target cell (E:T) ratio and the concentration of the construct. No killing of the A431 targets was seen in the absence of the construct, demonstrating that the TIL 660 line has no specificity for these cells. Significant levels of lysis were seen with concentrations of construct as low as 0.1 ng/ml (6×10^{-13}M), and this killing increased as a function of construct concentration or effector-to-target ratio. Very little additional killing was seen at concentrations above 25 ng/ml (1.5×10^{-10}M).

Exactly the same results were obtained when the constructs were made with the human Cγ1 or Cγ4 H-chain genes. The Cγ4 H chain was used for the construct because of its inability to fix human complement.

Additional tumor cell lines were tested for their susceptibility to TIL cell lysis in the presence of the anti-CD3/EGF constructs. These include a human metastatic melanoma line (M24) expressing a moderate level of EGF receptor, as well as a neuroblastoma line (IMR-32) that is very sensitive to lysis in an ADCC assay (lysis by Fc receptor-bearing cells in the presence of an anti-tumor antibody) but expresses little or no detectable EGF receptor. The results are shown in TABLE 1.

TABLE I

Cell Line	^{125}I-EGF Bound (pg/2 x 10^5 cells)
A431 (epidermal carcinoma)	236.8
M24 (metastatic melanoma)	34.1
IMR-32 (neuroblastoma)	0.72

The killing of these cell lines by the TIL 660 effectors was found to be directly related to the expression of EGF receptor (FIGS. 4B and 4C). The M24 line expresses EGF receptor, although ten-fold less than A431 cells, and is killed almost as well at low conjugate concentrations. The killing of A431 cells increased at higher concentrations of the conjugate (greater than 1.5 ng/ml) whereas the killing of M24 cells did not. This difference may reflect the saturation of M24 cell receptors at the lower concentration. The neuroblastoma line, IMR-32, does not express EGF receptor and was not killed by TIL 660 cells in the presence of the anti-CD3/EGF conjugate (FIG. 4C).

As shown in FIG. 5, a second cytotoxic T lymphocyte line, W-1, which is derived from peripheral blood and is both CD3+ and CD8+, also killed the EGF receptor-bearing A431 (FIG. 5A) and M24 (FIG. 5B) cells very efficiently in the presence but not in the absence of the construct.

The specific lysis of the A431 and M24 tumor cell lines was measured in the presence or absence of the conjugate, as well as its component parts. Four hour cytotoxicity assays were carried out using an effector (TIL 660 cells)-to-target ratio of 50:1 with the indicated additions. Values represent the amount of lysis obtained in a particular reaction expressed as the percentage of that obtained with the anti-CD3/EGF construct. The results are shown in TABLE 2.

TABLE 2

Line: Additions	% Maximum Lysis of Cell A431	M24
None	0	0
EGF (0.5 ng/ml)	0	0
Anti-CD3 (5 ng/ml)	0	0
EGF + Anti-CD3	1	0
Anti-CD3/EGF (5 ng/ml)	100	100
Construct + Anti-CD3 (0.5 μg/ml)	71	48
Construct + Anti-CD3 (10 μg/ml)	10	15

Neither EGF alone, anti-CD3 antibody alone, nor EGF in combination with anti-CD3 antibody were able to mediate cytotoxic T lymphocyte killing of the tumor targets. Concentrations of antibody that were 100-fold higher also did not significantly increase the specific lysis above background levels. Clearly, physical linkage of the antibody and EGF is required for killing activity since only the construct was able to mediate the lysis of the EGF receptor-bearing targets. Some inhibition of killing activity is possible with a 100-fold excess of anti-CD3 antibody. Since this represents only 0.5 μg/ml, it is possible that there may still be CD3 molecules available for binding. When the concentration was increased to 10 μg/ml, significant inhibition was observed.

The invention may be better understood from the following nonlimiting Examples, in which are described the preparation of bridging antibody fusion constructs using non-immunoglobulin binding agents chosen first from ligands adapted from the proteins EGF and CD4 and then from a single chain binding site adapted from the mouse anti-human melanoma antibody 9.2.27.

EXAMPLE 1

Constructs Utilizing Ligand Non-immunoglobulin Binding Agents

1. Plasmid Construction

An EGF gene fragment was synthesized from the known protein sequence described in Gregory et al. (J. Peptide Protein Res. (1977) 9:107-118), herein incorporated as reference. FIG. 2 and SEQ ID NO:1 shows the nucleic acid sequence synthesized and its corresponding amino acid sequence. A CD4 gene fragment (nucleic acid numbers 145-1266) encoding the extracellular domain including the variable-like region (amino acid numbers 1-94) and the joining-like region (amino acid numbers 95-109) was synthesized as described in Maddon et al. (Cell (1985) 92:93-104), herein incorporated as reference. The entire amino acid sequence including the transmembranous and cytoplasmic domains of the protein, along with its corresponding nucleic acid sequence, is shown in FIG. 3 and in SEQ ID NO:2.

The EGF or CD4 gene fragment was ligated to an engineered SmaI site at the 3' end of the human Cγ1 gene. This is shown schematically in FIG. 2. An XhoI site was placed to the 3' side of the EGF coding sequence for litigation to a fragment containing the 3' untranslated region and poly A addition signal from the mouse Ig CK gene.

V region cassettes encoding the H and L chain variable regions of the mouse anti-CD3 antibody, OKT3 (ATCC number CRL 8001), were constructed from cloned cDNAs as described by Gillies et al. (J. Immunol. Meth. (1989) 125:191-202), herein incorporated by reference. The cassettes were inserted into the chimeric antibody expression vector pdHL2 to give pdHL2-CD3. The modified H chain, to which EGF or CD4 was fused, was inserted into the pdHL2-CD3 plasmid as a HindIII to EcoRI fragment. A second construct was made by replacing the HindIII to NsiI fragment of the Cγ1 gene with the corresponding fragment of the Cγ4 gene. In both cases the lysine residue, normally found at the carboxy terminus of Ig H chains, was omitted from the fusion proteins.

2. Cell Culture and Transfection

Mouse hybridoma cells (Sp2/0 Ag14, ATCC No. CRL 1581) were maintained in Dulbecco's Modified Eagle's medium (DMEM) and transfected as described by Gillies et al. (Biotechnol. (1989) 7:799-804). Human tumor cell lines A431 (epidermal carcinoma, ATCC number CRL 1555), M24 (metastatic melanoma, originally obtained by D.C. Morton, UCLA, and provided by Ralph Reisfeld, Scripps Clinic), and IMR-32 (neuroblastoma, ATCC number CCL 127) were maintained in RPMI 1640 containing 10% FBS. The human tumor-infiltrating lymphocyte (TIL) line 660, derived from a human melanoma patient, was cultured in AIM V medium (GIBCO) containing IL2 (Hoffmann-LaRoche) as described by Reilly et al. (J. Immunol. Meth. (1990) 126:273-279). Greater than 90% of the cells were CD3+ and CD8+ when examined by fluorescence microscopy.

Transfectants secreting human antibody determinants were identified by ELISA, and their culture supernatants were tested further for anti-CD3 reactivity by their ability to stain TIL 660 cells in the presence of a fluorescenated anti-human Ig antiserum. Both the chimeric and conjugated antibody constructs were found to stain these cells as well as

the original mouse antibody (OKT3, Ortho Diagnostic Systems).

3. Protein Purification

Chimeric antibody, antibody/EGF constructs, and antibody/CD4 constructs were purified by affinity chromatography using protein A Sepharose (Repligen). Cell culture medium was used as a source of material for the purification. Electrophoretic analyses of the purified proteins showed that they were both fully assembled into antibody molecules and that the conjugated H chain migrated as would be expected for the fusion of the Ig and EGF sequences.

4. EGF Competitive Binding Assay

M24 melanoma cells (2×10^5 cells in a final volume of 0.1 ml) were mixed on ice in Hank's balanced salt solution containing 0.1% BSA and 20 mM HEPES together with ^{125}I-EGF (10 ng/ml final concentration, Amersham) and varying concentrations of cold competitor (either EGF, antibody or antibody conjugate). After a 2 hour incubation at 4°C, cells were washed three times by centrifugation, and the cell-associated radioactivity was counted. A non-specific background, determined by incubation with a 200-fold excess of cold EGF, was subtracted from all data points. The results were expressed as the percent inhibition of binding relative to the no-competitor control.

Alternatively, cells were incubated for 2.5 hours in 100 μl of buffer (HBSS, 0.1% BSA, 20 mM HEPES, pH 7.4) at 4°C with 700 pg of ^{125}I-EGF, washed three times with buffer and the pellet counted in a gamma counter. Non-specific binding (that obtained in the presence of a 200-fold excess of cold EGF) was subtracted.

5. Cytotoxicity Assay

Cytotoxicity assays were carried out using ^{51}Cr-labeled tumor targets and TIL 660 cells as effectors. A fixed number

of labeled targets (10^4 per well) in 50 μl and varying numbers of effectors in 50 μl were mixed with 100 μl of diluted antibody or conjugate in the wells of a microtiter plate. The plates were centrifuged and assayed for released ^{51}Cr following a 4 hr incubation at 37°C. Spontaneous release was subtracted from experimental values and the percent of specific lysis was determined by dividing the corrected release value by the total released with detergent lysis.

The assay for activity of Ig/CD4 constructs may be carried out in a manner analogous to that for Ig/EGF constructs, with the modification that the target cells used would be those expressing gp120 on their surfaces, such as HIV-infected cells or cells that have been transfected with a gene for gp120 and are expressing it on their surfaces.

EXAMPLE 2

Construct Utilizing A Single Chain Binding Site Non-Immunoglobulin Binding Agent

1. Plasmid Construction

The V_L and V_H regions from the mouse antibody 9.2.27 (described by Beavers et al. in the published European patent application No. 411893, published February 6, 1991), specific for a human melanoma-specific proteoglycan antigen, were adapted using the polymerase chain reaction (PCR) technique to form a single-chain binding site-encoding sequence. Native 9.2.27 sequences were modified by the addition of 5' and 3' primers. Primers added to the 5' end of each V region were identical to the "sense" strand of the DNA encoding the first six amino acid residues of the mature H and L proteins. Upstream of these were provided sequences encoding a BglII restriction site for subsequent joining steps, and an EcoRI site for use in cloning the PCR products. Likewise, primers derived from the 3' end of each V region (in this case anti-

sense primers) were identical to the last six amino acids of each. Additional sequences were added for cloning purposes and for either joining purposes (for the V_L region) or to introduce a stop codon and a convenient XhoI restriction site (in the V_H region). A carboxyl-terminal Lys was added to the end of V_H since all antibody H chains end with this amino acid.

The sequences of the sense and anti-sense primers were as follows:

9.2.27 5' L chain sense primer:

```
5'-CGGAGAATTCAGATCT AAC ATT GTG CTG ACC CAA-3'
        '----''----'Asn Ile Val Leu Thr Gln
         EcoRI BglII
```

9.2.27 3' L chain anti-sense primer:

```
5'--TTTGTCGA CTT TAT TTC CAA CTT TGT C-3'
        '----'Lys Ile Glu Leu Lys Thr
         SalI
```

9.2.27 5' H chain sense primer:

```
5'-CCCGAATTCAGATCT CAG GTC CAG CTG CAG CAG-3'
       '----''----' Gln Val Gln Leu Gln Gln
        EcoRI BglII
```

9.2.27 3' H chain anti-sense primer:

```
5'-CGCCCTCGTG TCA CTT TGA GGA GAC GGT GAC TGA GG-3'
        '----'STOP Lys Ser Ser Val Thr Val Ser
         XhoI
```

Underlined portions of the above sequences are those which are homologous to the original 9.2.27 V regions. The coding of each codon in the above anti-sense primers is shown in reverse and represents the non-coding strand; e.g., CTT in the above anti-sense primer shown 5' to 3' corresponds to the coding sequence AAG (Lys).

The V_L and V_H PCR products were synthesized by mixing 1 ng of template (a plasmid containing both V regions) with 50 ng of each set of primers in 100 μL standard PCR reactions

(Perkin Elmer/Cetus). These products were digested within EcoRI and Sal I (for V_L) or EcoRI and XhoI (for V_H). The V_H product was cloned as an EcoRI-to-XhoI fragment and verified by DNA sequencing. The V_L region was ligated to the 5' end of a synthetic linker fragment encoding a 5' XhoI site, a flexible peptide linker composed of Ser and Gly residues, and a 3' BamHI site:

```
5'-(C)TCGAGCGGGGGCAGCGGGGGCGGAGGCAGCGGCGGGGGCG-3'
        CGCCCCCGTCGCCCCCGCCTCCGTCGCCGCCCCCGCCTAG(G)
   '------'                                    "------"
    XhoI                                        BamHI
```

and cloned as an EcoRI-to-BamHI fragment (the XhoI and SalI site having compatible ends). After verification of the V_L-linker sequence, the cloned V_H fragment was digested with BglII and XhoI and joined to the V_L-linker fragment at the 3' BamHI site (BglII and BamHI having compatible ends).

The joining of the V_L and V_H segments via their respective Sal I and BglII sites with the XhoI-to-BamHI linker fragment is illustrated in FIG. 6A. These restriction sites became non-functional after they were ligated, the protein sequences encoded by these restriction sites being composed of either Gly or Ser.

The resulting 9.2.27 V_L-linker-V_H sequence, herein referred to as 9.2.27sca, was joined to the CH3 exon of the human Cγ4 gene by first modifying the 3' end of the CH3 exon to encode a BamHI site. A short oligonucleotide (GGGATCCC) was ligated to the SmaI site near the end of the CH3, changing the 3' end sequence from

```
  SmaI                          BamHI
                              SmaI '-----'
'--------'                  '--------'
C CCG GGA AAA      to       C CCG GGA TCC   .
  Pro Gly Lys                 Pro Gly
```

9.2.27sca was joined to this CH3 BamHI site via its unique 5' BglII site resulting in the addition of a single Ser residue. The Cγ4-9.2.27sca fusion protein coding sequence was then inserted into a pdHL2 chimeric antibody expression vector containing the V regions of the anti-CD3 antibody, as described in Example 1 and shown in FIG. 6B. A poly-A addition site (pA) was provided by the vector and, in the completed vector, was located to the 3' side of the translation stop signal in the 9.2.27 V_H region.

2. Production of Construct and Cytotoxicity Assay

Cell culture and transfection with the above vector, protein purification of the resulting proteins, and cytotoxicity assays using those proteins were carried out in the same manner as with the fusion proteins of Example 1. Figure 7 shows the results of a killing assay using varying concentrations of the anti-CD3/9.2.27sca bridging antibody and varying effector-to-target ratios of TIL 660 (effector) and M21 melanoma (target) cells. Significant killing of target cells occurred at relatively low effector-to-target ratios; this killing was seen to increase with the concentration of bridging antibody.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Gillies, Stephen D.

(ii) TITLE OF INVENTION: Bridging Antibody Fusion Constructs

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS

(A) ADDRESSEE: Abbott Laboratories

(B) STREET: One Abbott Park Road, D-377,AP6D

(C) CITY: Abbott Park

(D) STATE: Illinois

(E) COUNTRY: U.S.A.

(F) ZIP: 60064-3500

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 720kb storage

(B) COMPUTER: IBM XT

(C) OPERATING SYSTEM: DOS 3.30

(D) SOFTWARE: Word Perfect 5.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(2) INFORMATION FOR SEQ ID NO. 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 175 nucleic acids
53 amino acids

(B) TYPE: nucleic acid, amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA, protein

(iii) HYPOTHETICAL: DNA, yes; protein, no
(iv) ANTI-SENSE: no
(vi) ORIGINAL SOURCE
(A) ORGANISM:-human
(B) TISSUE TYPE: serum
(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(ix) SEQUENCE DESCRIPTION: SEQ ID No: 1

```
C CCG GGT AAC TCC GAC TCT GAA TGT CCC CTG                31
  Pro Gly Asn Ser Asp Ser Glu Cys Pro Leu
        +1                5

AGC CAC GAC GGC TAC TGC CTG CAC GAC GGC                  61
Ser His Asp Gly Tyr Cys Leu His Asp Gly
     10                  15

GTG TGC ATG TAC ATC GAG GCC CTG GAC AAG                  91
Val Cys Met Tyr Ile Glu Ala Leu Asp Lys
     20                  25

TAC GCATGC AAC TGC GTG GTC GGG TAC ATC                   121
Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile
     30                  35

GGC GAG AGG TGC CAG TAC AGG GAC CTC AAG                  151
Gly Glu Arg Cys Gln Tyr Arg Asp Leu Lys
     40                  45

TGG TGG GAG CTC CGG TGACTCGAG                            175
Trp Trp Glu Leu Arg
     50
```

(2) INFORMATION FOR SEQ. ID NO: 2
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1742 base pairs, 446 amino acids
(B) TYPE: nucleic acid, amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: CDNA, protein
(iii) HYPOTHETICAL: no
(iv) ANTI-SENSE: no
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human/mouse
(B) TISSUE SOURCE: blood
(vii) IMMEDIATE SOURCE: T cell
(A) LIBRARY: Charon 4 human genomic
(ix) SEQUENCE DESCRIPTION: SEQ ID No: 2

```
CAAGCCCAGA GCCCTGCCAT TTCTGTGGGC TCAGGTCCCT                    40

ACTGCTCAGC CCCTTCCTCC CTCGGCAAGG CCACA ATG                     78
                                        met

AAC CGG GGA GTC CCT TTT AGG CAC TTG CTT CTG                    111
Asn Arg Gly Val Pro Phe Arg His Leu Leu Leu
         -20                 -15

GTG CTG CAA CTG GCG CTC CTC CCA GCA GCC ACT                    144
Val Leu Gln Leu Ala Leu Leu Pro Ala Ala Thr
    -10                 -5

CAG GGA AAC AAA GTG GTG CTG GGC AAA AAA GGG                    177
Gln Gly Asn Lys Val Val Leu Gly Lys Lys Gly
+1               5                  10

GAT ACA GTG GAA CTG ACC TGT ACA GCT TCC CAG                    210
Asp Thr Val Glu Leu Thr Cys Thr Ala Ser Gln
             15                  20

AAG AAG AGC ATA CAA TTC CAC TGG AAA AAC TCC                    243
```

```
Lys Lys Ser Ile Gln Phe His Trp Lys Asn Ser
        25                  30

AAC CAG ATA AAG ATT CTG GGA AAT CAG GGC TCC          276
Asn Gln Ile Lys Ile Leu Gly Asn Gln Gly Ser
     35                  40

TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT          309
Phe Leu Thr Lys Gly Pro Ser Lys Leu Asn Asp
45                  50                  55

CGC GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA          342
Arg Ala Asp Ser Arg Arg Ser Leu Trp Asp Gln
                    60                  65

GGA AAC TTC CCC CTG ATC ATC AAG AAT CTT AAG          375
Gly Asn Phe Pro Leu Ile Ile Lys Asn Leu Lys
             70                  75

ATA GAA GAC TCA GAT ACT TAC ATC TGT GAA GTG          408
Ile Glu Asp Ser Asp Thr Tyr Ile Cys Glu Val
         80                  85

GAG GAC GAG AAG GAG GAG GTG GAA TTG CTA GTG          441
Glu Asp Gln Lys Glu Glu Val Gln Leu Leu Val
     90                  95

TTC GCA TTG ACT GCC AAC TCT GAC ACC CAC CTG          474
Phe Gly Leu Thr Ala Asn Ser Asp Thr His Leu
100                 105                 110

CTT CAG GGG GAG AGC CTG ACC CTG ACC TTG GAG          507
Leu Gln Gly Gln Ser Leu Thr Leu Thr Leu Glu
                115                 120
```

```
AGC CCC CCT GGT AGT AGC CCC TCA GTG CAA TGT          540
Ser Pro Pro Gly Ser Ser Pro Ser Val Gln Cys
            125                 130

AGG AGT CCA AGG GGT AAA AAC ATA CAG GGG GGG          573
Arg Ser Pro Arg Gly Lys Asn Ile Gln Gly Gly
        135                 140

AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG          606
Lys Thr Leu Ser Val Ser Gln Leu Glu Leu Gln
    145                 150

GAT AGT GGC ACC TGG ACA TGC ACT GTC TTG CAG          639
Asp Ser Gly Thr Trp Thr Cys Thr Val Leu Gln
155                 160                 165

AAC CAG AAG AAG GTG GAG TTC AAA ATA GAC ATC          672
Asn Gln Lys Lys Val Glu Phe Lys Ile Asp Ile
                170                 175

GTG GTG CTA GCT TTC CAG AAG GCC TCC AGC ATA          705
Val Val Leu Ala Phe Gln Lys Ala Ser Ser Ile
            180                 185

GTC TAT AAG AAA GAG GGG GAA CAG GTG GAG TTC          738
Val Tyr Lys Lys Glu Gly Glu Gln Val Glu Phe
        190                 195

TCC TTC CCA CTC GCC TTT ACA GTT GAA AAG CTG          771
Ser Phe Pro Leu Ala Phe Thr Val Glu Lys Leu
    200                 205

ACG GGC AGT GGC GAG CTG TGG TGG CAG GCG GAG          804
Thr Gly Ser Gly Glu Leu Trp Trp Gln Ala Glu
210                 215                 220
```

```
AGG GCT TCC TCC TCC AAG TCT TGG ATC ACC TTT                    837
Arg Ala Ser Ser Ser Lys Ser Trp Ile Thr Phe
                225                 230

GAC CTG AAG AAC AAG GAA GTG TCT GTA AAA CGG                    870
Asp Leu Lys Asn Lys Glu Val Ser Val Lys Arg
            235                 240

GTT ACC CAG GAC CCT AAG CTC CAG ATG GGC AAG                    903
Val Thr Gln Asp Pro Lys Leu Gln Met Gly Lys
        245                 250

AAG CTC CCG CTC CAG CTC ACC CTG CCC CAG GCC                    936
Lys Leu Pro Leu His Leu Thr Leu Pro Gln Ala
    255                 260

TTG CCT CAG TAT GCT GGC TCT GGA AAC CTC ACC                    969
Leu Pro Gln Tyr Ala Gly Ser Gly Asn Leu Thr
265                 270                 275

CTG GCC CTT GAA GCG AAA ACA GGA AAG TTG CAT                    1002
Leu Ala Leu Glu Ala Lys Thr Gly Lys Leu His
                280                 285

CAG GAA GTG AAC CTG GTG GTG ATG AGA GCC ACT                    1035
Gln Gln Val Asn Leu Val Val Met Arg Ala Thr
            290                 295

CAG CTC CAG AAA AAT TTG ACC TGT GAG GTG TGG                    1068
Gln Leu Gln Lys Asn Leu Thr Cys Glu Val Trp
        300                 305

GGA CCC ACC TCC CCT AAG CTG ATG CTG AGC TTG                    1101
Gly Pro Thr Ser Pro Lys Leu Met Leu Ser Leu
    310                 315
```

```
AAA CTG GAG AAC AAG GAG GCA AAC GTC TCG AAG          1134
Lys Leu Glu Asn Lys Glu Ala Lys Val Ser Lys
320                 325                 330

CGG GAG AAG GCG GTG TGG GTG CTG AAC CCT GAG          1167
Arg Glu Lys Ala Val Trp Val Leu Asn Pro Glu
                335                 340

GCG GGG ATG TGG CAG TGT CTG CTG AGT GAC TCG          1200
Ala Gly Met Trp Gln Cys Leu Leu Ser Asp Ser
            345                 350

GGA CAG GTC CTG CTG GAA TCC AAC ATC AAG GTT          1233
Gly Gln Val Leu Leu Glu Ser Asn Ile Lys Val
        355                 360

CTG CCC ACA TGG TCC ACC CCG GTG CAG CCA ATG          1266
Leu Pro Thr Trp Ser Thr Pro Val Gln Pro Met
    365                 370

GCC CTG ATT GTG CTG GGG GGC GTC GCC GGC CTC          1299
Ala Leu Ile Val Leu Gly Gly Val Ala Gly Leu
375                 380                 385

CTG CTT TTC ATT GGG CTA GGC ATC TTC TTC TGT          1332
Leu Leu Phe Ile Gly Leu Gly Ile Phe Phe Cys
                390                 395

GTC AGG TGC CGG CAC CGA AGG CGC CAA GCA GAG          1365
Val Arg Cys Arg His Arg Arg Arg Gln Ala Glu
            400                 405

CGG ATG TCT CAG ATC AAG AGA CTC CTC AGT GAG          1398
Arg Met Ser Gln Ile Lys Arg Leu Leu Ser Glu
        410                 415
```

```
AAG AAG ACC TGC CAG TGC CCT CAC CGG TTT CAG          1431
Lys Lys Thr Cys Gln Cys Pro His Arg Phe Gln
    420                 425

AAG ACA TGT AGC CCC ATT TGA GGCACGAGGC CAGG          1466
Lys Thr Cys Ser Pro Ile ---
430                 435

CAGATCCCAC TTGCAGCCTC CCCAGGTGTC TGCCCCGCGT          1506
TTCCTGCCTG CGGACCAGAT GAATGTAGCA GATCCCACGC          1546
TCTGGCCTCC TGTTCGTCCT CCCTACAATT TGCCATTGTT          1586
TCTCCTGGGT TAGGCCCCGG CTTCACTGGT TGAGTGTTGC          1626
TCTCTAGTTT CCAGAGGCTT AATCACACCG TCCTCCACGC          1666
CATTTCCTTT TCCTTCAAGC CTAGCCCTTC TCTCATTATT          1706
TCTCTCTGAC CCTCTCCCCA CTGCTCATTT GGATCC              1742
```

We claim:

1. A nucleic acid sequence encoding an amino acid sequence comprising:

(a) a heavy chain variable region which, when combined with a light chain variable region, binds to a surface antigen on an effector cell;

(b) a heavy chain constant region comprising a C_{H3} domain; and

(c) a non-immunoglobulin binding agent which binds a surface protein on a target cell.

2. The nucleic acid sequence of Claim 1 wherein one of said non-immunoglobulin binding agent and said surface protein comprises a ligand and the other comprises a receptor which has an affinity for said ligand.

3. The nucleic acid sequence of Claim 2 wherein said non-immunoglobulin binding agent comprises a ligand selected from the group consisting of a hormone, an active hormone analog, an active hormone fragment, a growth factor, an active growth factor analog, and an active growth factor fragment.

4. The nucleic acid sequence of Claim 3 wherein said ligand is selected from the group consisting of epidermal growth factor (EGF), and said receptor comprises the EGF receptor.

5. The nucleic acid sequence of Claim 1 wherein said heavy chain variable region binds with a surface antigen on an effector cell selected from the group consisting of cytotoxic T lymphocytes, macrophages, monocytes, large granular lymphocytes, eosinophils, and natural killer cells.

6. The nucleic acid sequence of Claim 1 wherein said non-immunoglobulin binding agent comprises a single chain binding site.

7. The nucleic acid sequence of Claim 6 wherein said single chain binding site is adapted from a variable region of a mammalian antibody.

8. A cell line transfected with the nucleic acid sequence of Claim 1.

9. The cell line of Claim 8 wherein said cell line is selected from the group consisting of myeloma and hybridoma cell lines.

10. A method of producing a bridging antibody construct comprising the steps of:

(a) linking nucleic acid sequences encoding amino acid sequences including:

(i) a heavy chain variable region which, when-combined with a light chain variable region, binds to a surface antigen on an effector cell;

(ii) a heavy chain constant region comprising a C_{H3} domain; and

(iii) a non-immuhoglobulin binding agent which binds a surface protein on a target cell;

(b) transfecting a host cell with said nucleic acid sequence; and

(c) culturing said transfected cell such that it expresses said construct.

11. A bridging antibody construct encoded by the nucleic acid sequence of Claim 1.

12. A method of selectively killing a target cell in vivo comprising the steps of:

(a) providing a bridging antibody construct comprising:

(i) a heavy chain variable region which, when combined with a light chain variable region, binds to a surface antigen on an effector cell;

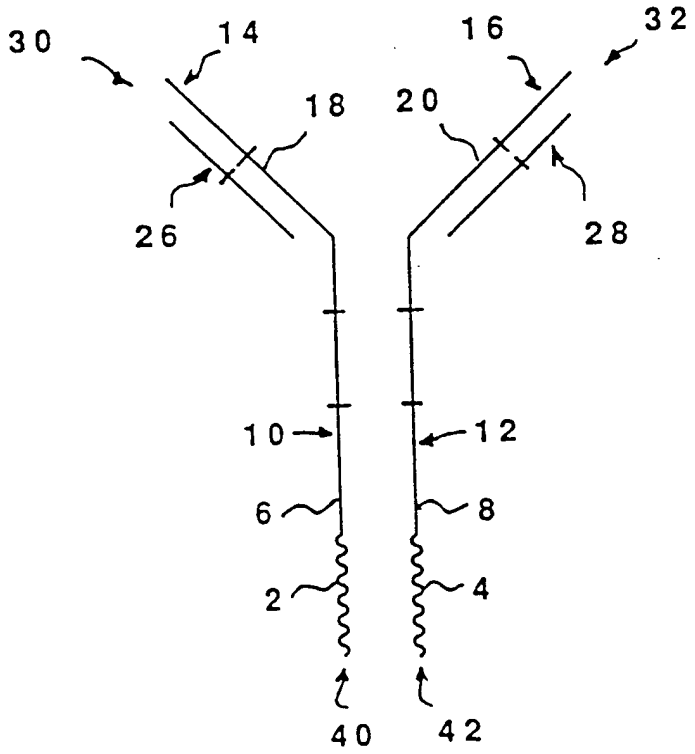
(ii) a heavy chain constant region comprising a C_{H3} domain; and

(iii) a non-immunoglobulin binding agent which binds a surface protein on a target cell, said agent being peptide-bonded to the carboxy terminus of said C_{H3} domain; and

(b) administering a therapeutically affective amount of said construct to the circulation of a subject harboring said target cell, said construct bringing said effector cell in contact with said target cell and thereby killing or neutralizing said target cell.

1/7

Figure 1



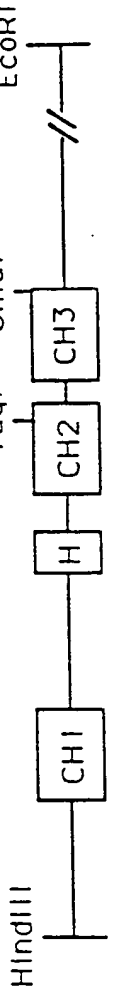


Figure 2A

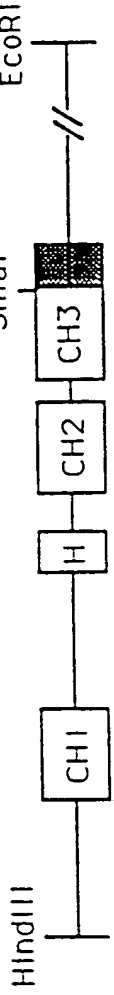


Figure 2B

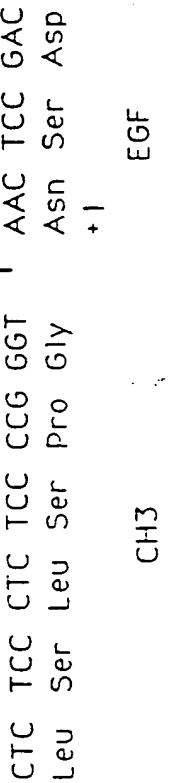


Figure 2C

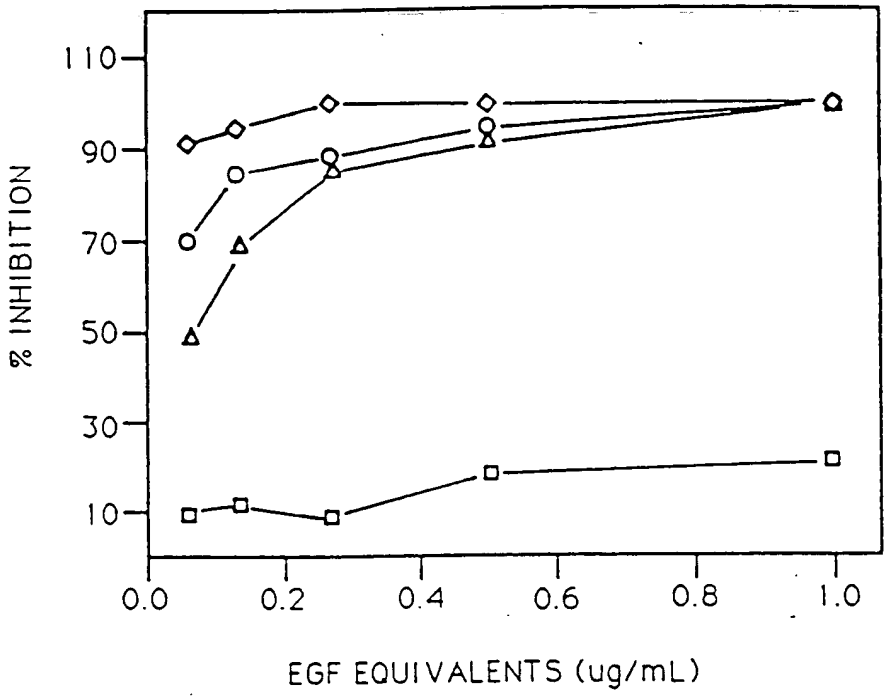

110
90
70
50
30
10
% INHIBITION
0.0
0.2
0.4
0.6
0.8
1.0
EGF EQUIVALENTS (ug/mL)
Construct
Unlabeled EGF
Anti-CD3 Ab
Anti-EGF Receptor Ab

Figure 3

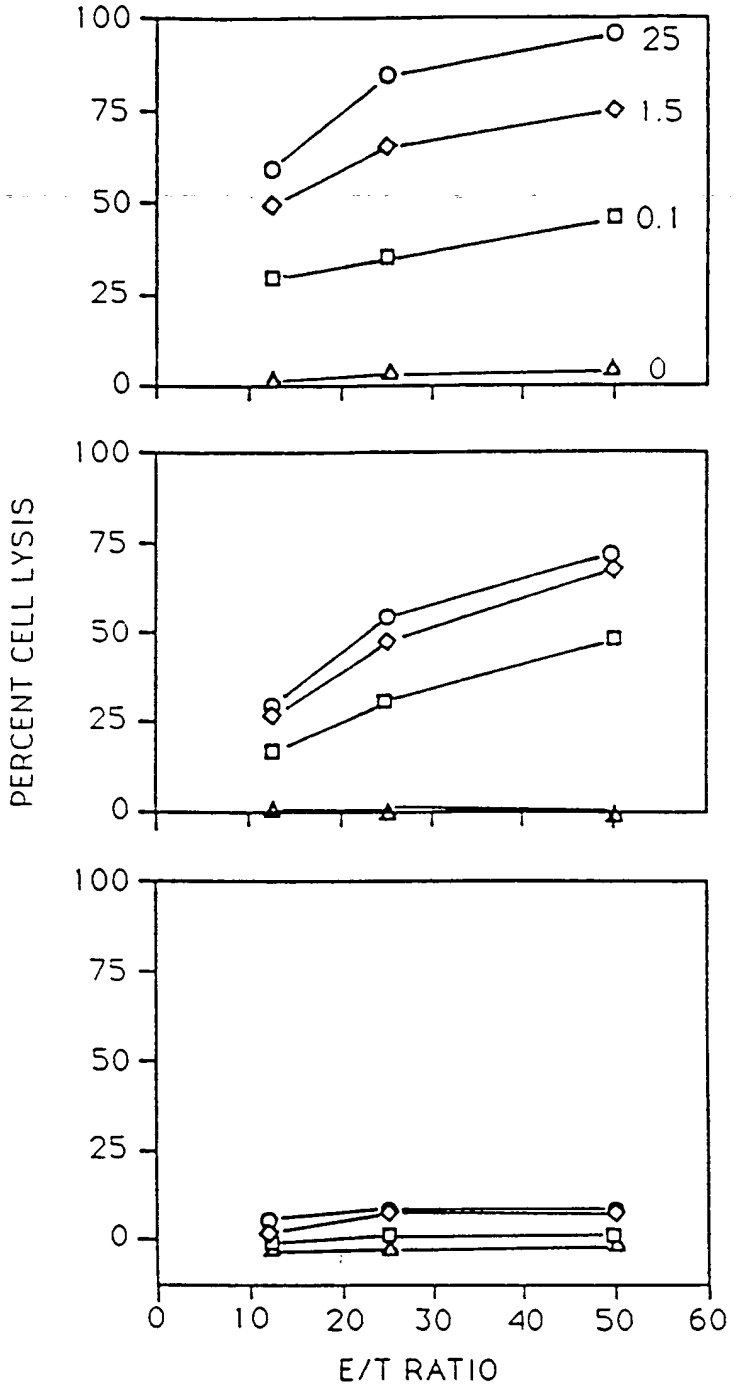


Figure 4A

Figure 4B

Figure 4C

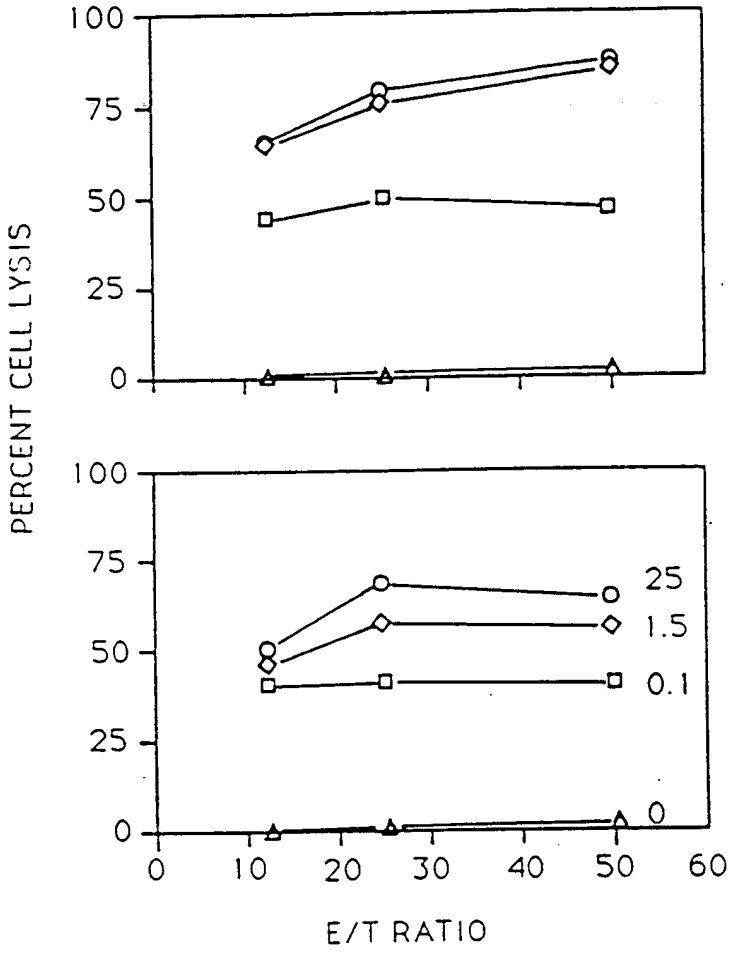


Figure 5A

Figure 5B

Figure 6A

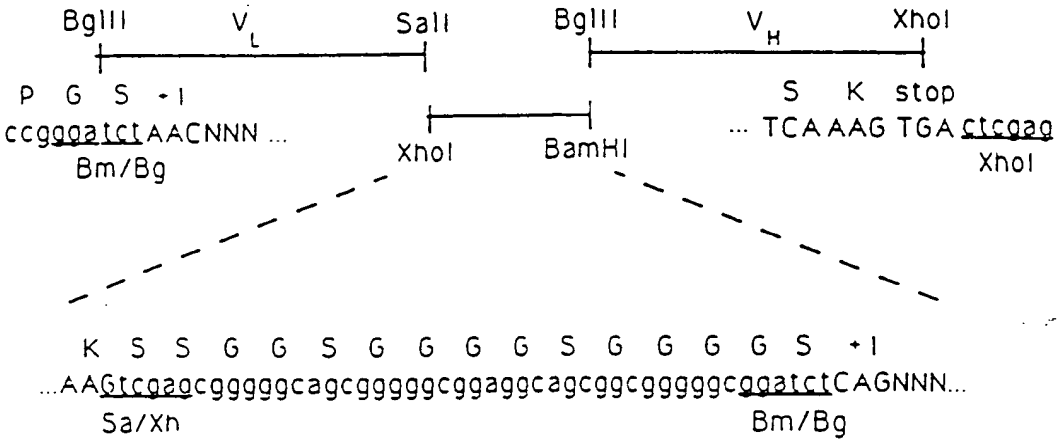


Figure 6B

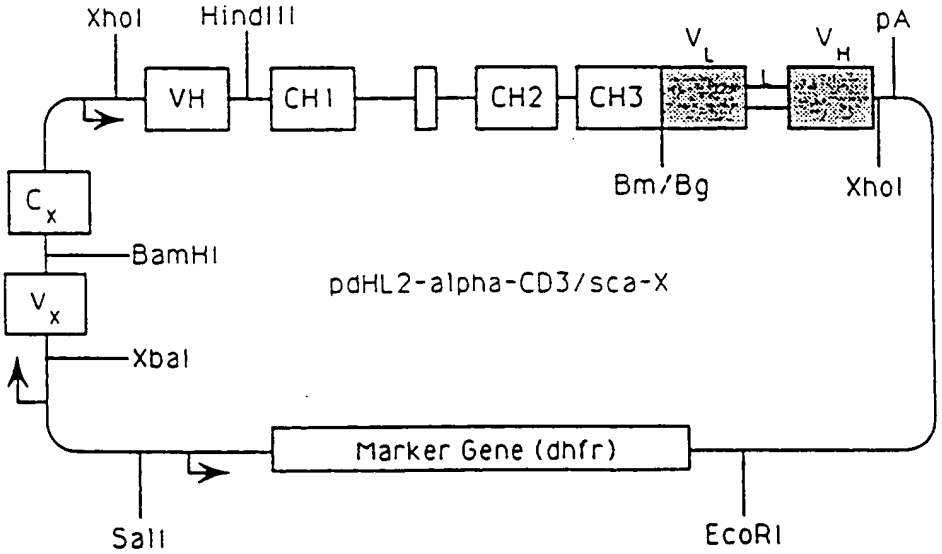
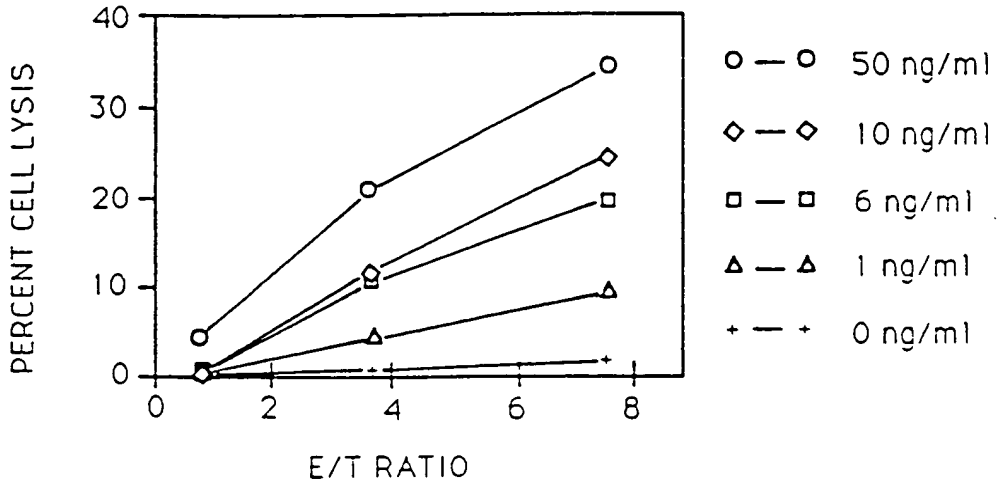


Figure 7


PERCENT CELL LYSIS
40
30
20
10
0
0
2
4
6
8
E/T RATIO
50 ng/ml
10 ng/ml
6 ng/ml
1 ng/ml
0 ng/ml

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08421

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 21/06; C12N 15/00; A61K 35/14; C07K 3/00
US CL : 530/387; 424/85.8; 435/69.6, 320.1

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/387;424/85.8;435/69.6,320.1

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Science, Volume 239, issued 22 January 1988, Margaret Ann Liu et. al, "Hormone Conjugated with Antibody to CD3 mediates Cytotoxic T Cell Lysis of Human Melanoma Cells", pages 395-398, see 396 and 397.	1-12
Y	Nature, Volume 337, issued 09 February 1989, Daniel J. Capon et al, "Designing CD4 Immunoadhesions for AIDS Therapy", pages 525-530, see entire document.	1-12
Y	US, A, 4,816,567 (Cabilly et al) 28 March 1989, see columns 7, 16 and 17.	1-12
Y	Proceeding of the National Academy of Sciences, Volume 87, issued July 1990, Seung Uon Shin et al, "Expression and Characterization of an Antibody Binding Specificity Joined to Insulin like Growth Factor 1: Potential Applications for Cellular Targeting", pages 5322-5326, see entire document.	1-12
Y	Nucleic Acids Research, Volume 17, No. 24, issued 1989, Clackson et al, "'Sticky feet'- Directed Mutagenesis and its Application to Swapping Antibody Domains", pages 10163-10170, see pages 10164-10166.	1-12

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
05 FEBRUARY 1992	18 FEB 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Lila Feisee

Form PCT/ISA/210 (second sheet)(May 1986) 8

0556328

Application Number
EP 92 90 1152

Office

C07K16/46B Y

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
P,X	WO-A-91 14438 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 3 October 1991 * the whole document *	1-12	C12N15/62 C07K15/00 C12N15/10 A61K37/02
Y	WO-A-89 05816 (PROTEIN DESIGN LABS, INC.) 29 June 1989 * the whole document *	1-12	
Y	WO-A-88 09344 (CREATIVE BIOMOLECULES, US) 1 December 1988 * page 15, line 18 - page 18, line 6; figures 2,8 * * page 67, paragraph IV *	1-12	
Y	EP-A-0 394 827 (HOFFMANN - LA ROCHE AG) 31 October 1990 * the whole document *	1-12	
A	EP-A-0 396 387 (RESEARCH DEVELOPMENT FOUNDATION) 7 November 1990		

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C12N
C07K

The supplementary search report has been drawn up for the claims attached hereto.

Place of search	Date of completion of the search	Examiner
THE HAGUE	12 April 1994	Nauche, S

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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mod.

CLAIMS

1. A nucleic acid sequence encoding an amino acid sequence comprising:

(a) a heavy chain variable region which, when combined with a light chain variable region, binds to a surface antigen on an effector cell;

(b) a heavy chain constant region comprising a C_{H3} domain; and

(c) a non-immunoglobulin binding agent which binds a surface protein on a target cell.

2. A nucleic acid sequence as claimed in claim 1 wherein one of the non-immunoglobulin binding agent and the surface protein comprises a ligand and the other comprises a receptor which has an affinity for the ligand.

3. A nucleic acid sequence as claimed in claim 2 wherein the non-immunoglobulin binding agent comprises a ligand which is a hormone, an active hormone analogue, an active hormone fragment, a growth factor, an active growth factor analogue or an active growth factor fragment.

4. A nucleic acid sequence as claimed in claim 3 wherein the ligand is epidermal growth factor (EGF) and the receptor comprises the EGF receptor.

5. A nucleic acid sequence as claimed in any one of claims 1 to 4, wherein the heavy chain variable region binds with a surface antigen on an effector cell which is a cytotoxic T lymphocyte, macrophage, monocyte, large granular lymphocyte, eosinophil or natural killer cell.

6. A nucleic acid sequence as claimed in any one of claims 1 to 5 wherein the non-immunoglobulin binding agent comprises a single chain binding site.

7. A nucleic acid sequence as claimed in claim 6, wherein the single chain binding site is adapted from a variable region of a mammalian antibody.

8. A cell line transfected with a nucleic acid sequence as claimed in any one of claims 1 to 7.

9. A cell line as claimed in claim 8, wherein the cell line is a myeloma or hybridoma cell line.

10. A method of producing a bridging antibody construct comprising the steps of:

(a) linking nucleic acid sequences encoding amino acid sequences including:

(i) a heavy chain variable region which, when-combined with a light chain variable region, binds to a surface antigen on an effector cell;

(ii) a heavy chain constant region comprising a C_{H3} domain; and

(iii) a non-immunoglobulin binding agent which binds a surface protein on a target cell;

(b) transfecting a host cell with the linked nucleic acid sequence; and

(c) culturing the transfected cell such that it expresses the construct.

11. A bridging antibody construct encoded by a nucleic acid sequence as claimed in any one of claims 1 to 7.

12. The use of a bridging antibody construct comprising:

(i) a heavy chain variable region which, when combined with a light chain variable region, binds to a surface antigen on an effector cell;

(ii) a heavy chain constant region comprising a C_{H3} domain; and

(iii) a non-immunoglobulin binding agent which binds a surface protein on a target cell, the agent being peptide-bonded to the carboxy terminus of the C_{H3} domain;

in the preparation of an agent for selectively killing a target cell *in vivo*.